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(57) Abstract

The present invention discloses a DNA sequence encoding the exo-polygalacturonase gene from *Aspergillus*. Specifically the *Aspergillus tubigensis* exo-polygalacturonase gene is cloned and expressed. The invention relates to vectors comprising the exo-polygalacturonase coding sequence and to host cells transformed with such vectors. The invention further relates to the production of recombinant exo-polygalacturonase and to the use of this protein.

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Cloning and expression of the exo-polygalacturonase gene from Aspergillus

Technical field

The present invention is in the field of molecular biology. Specifically, a DNA sequence encoding the exo-polygalacturonase gene from Aspergillus is cloned and expressed. The invention relates to vectors comprising the exo-polygalacturonase coding sequence and to host cells transformed with such vectors. The invention further relates to the production of recombinant exo-polygalacturonase. Furthermore, as an example of the application of this enzyme partial pectin degradation is demonstrated.

Background of the invention

Pectins are major constituents of the cell walls of edible parts of fruits and vegetables. The middle lamella which are situated between the cell walls are mainly built up from protopectin which is the insoluble form of pectin. Pectins are considered as intercellular adhesives and due to their colloid nature they also have an important function in the water-regulation of plants. Water-binding capacity is greatly increased by the amount of hydrophylic hydroxyl and carboxyl groups. The amount of pectin can be very high. For example, lemon peels are reported to contain pectin up to 30% of their dry weight, orange peels contain from 15-20% and apple peels about 10% (Norz, K., 1985. Zucker und Süsswaren Wirtschaft 38 5-6).

Pectins are composed of a rhamno-galacturonan backbone in which 1,4-linked α-D-galacturonan chains are interrupted at intervals by the insertion of 1,2-linked α-L-

rhamnopyranosyl residues (Pilnik, W. and A. Voragen 1970. In 'The Biochemistry of fruits and their products', Vol. 1, Chapter 3, p.53. Acad. Press). Other sugars, such as D-galactose, L-arabinose and D-xylose, are present as side chains. A large part of the galacturonan residues is esterified with methyl groups at the C2 and C3 position.

Pectin-degrading enzymes are important tools in the food industry. Traditionally these enzymes are used as mixtures. Aspergillus niger and other fungi produce a whole spectrum of enzymes which can advantageously be used in the degradation of pectin. Examples of such enzymes are pectin esterase, pectin lyase (also called pectin transeliminase), endo- and exo-polygalacturonases. In A. niger the pectin degrading proteins are not expressed constitutively. Induction of these enzymes is achieved by growing the strains when carbon sources such as glucose or sucrose are limiting and in the presence of pectin or breakdown products thereof.

In order to avoid the problem of induction and also to avoid obtaining not well-defined enzyme mixtures there is a growing tendency to clone the genes encoding these enzymes and to express them in other more suitable host cells.

The cloning and expression of several of these enzymes obtained from Aspergillus niger has been reported. EP 0 278 355 describes the cloning of the pectin lyase gene, the sequence thereof and the expression. EP 0 353 188 adds some other pectin lyases.

As mentioned above pectin contains a backbone comprising a high amount of 1,4-linked α-D-galacturonan molecules. This backbone can be digested by the action of depolymerases. Two such depolymerases are known, endo- and exo-polygalacturonase.

Examples of the cloning and expression of especially the former one of these depolymerases have been reported.

EP 0 421 919 discloses two polygalacturonases which can be classified as endo-polygalacturonases. Another endo-polygalacturonase has been disclosed in EP 0 388 593. Both of these patent application used Aspergilli as the source of the gene.

Exo-polygalacturonases are not so abundant as the endo-form as evidenced by the reports about this enzyme published so far.

Mill (Biochem. J. 99 : 557-561 and 562-565 (1966)) reported the isolation and characterization of two distinct exo-polygalacturonases. One of these was found to be mercury activated. This was confirmed by Hara et al. (Nippon Shokuhim Kogyo Gakkashi 31 : 581-586 (1984)). Kester and Visser (Biotechn. Appl. Biochem, 12 : 150-160 (1990)) report the presence of only one exo-polygalacturonase and 5 endo-polygalacturonases in A. niger culture filtrates. Finally, the cloning of exo-polygalacturonase from Erwinia chrysanthemi EC16 has been reported (He and Collmer J. Bacteriol. 172 : 4988-4995 (1990)).

Exo-polygalacturonase is capable of converting polygalacturonides to galacturonic acid. To enhance this process it is advantagous to use pure exo-polygalacturonase. Galacturonic acid can be used as a source for different synthetic reactions it has for example been found to be useful in the production of ascorbic acid.

Summary of the invention

The present invention provides an isolated recombinant DNA sequence encoding exo-polygalacturonase. Preferably the exo-polygalacturonase is obtained from Aspergilli, more preferably it is obtained from Aspergillus tubigensis or from Aspergillus niger.

The invention also provides an expresssion cassette containing a DNA sequence encoding the exo-polygalacturonase. Such an expression cassette contains appropriate regulatory regions i.e. a promoter, optionally an enhancer, a terminator. The cassette may also contain a selection marker encoding gene.

In a further aspect of the invention the expression cassette forms part of a vector.

The invention also provides recombinant host cells transformed with the vector containing the exo-polygalacturonase encoding DNA.

The invention further provides isolated recombinant exo-polygalacturonase.

The invention also discloses a method for the production of exo-polygalacturonases. The invention shows how the exo-polygalacturonase can be purified.

The invention further discloses a method for obtaining galacturonic acid comprising the use of exo-polygalacturonase.

Finally, the invention describes the use of exo-polygalacturonase in the treatment of pectin.

Description of the Figures

Figure 1 shows the oligonucleotide mixtures derived from N-terminal amino acid sequences from cyanogen bromide fragments of A. tubigensis exo-polygalacturonase PGX. These DNA probes have been used in hybridization experiments to detect the pgaX gene.

Figure 2 shows the restriction map of the A. tubigensis pgaX gene. The open arrow indicates the location and orientation of the gene.

Figures 3 and 4 show the results of the use of exo-PG versus Rohament PL for reducing the calcium sensitivity of pectin. X-axis shows molecular weight change (in kD) and Y-axis shows viscosity (in centipoise). The dosages of enzyme in mg per g pectin are printed next to the data points.

o = Rohament PL, + = exo-PG ⌧ = average of blanks.

Detailed description of the invention

The present invention discloses purified and isolated DNA sequences which encode a polypeptide having the activity of the Aspergillus tubigensis PGX enzyme the seqence is characterized in that it comprises the sequence of SEQ ID No.1, genetic variants of the sequence of SEQ ID N0 1 are likewise considered to be part of the invention as are DNA sequences encoding for the same amino acid sequence but altered making use of the degeneracy of the genetic code. Further included in the present invention are DNA sequences capable of hybridizing with the sequence of SEQ ID No. 1 or with parts thereof.

The invention also discloses DNA constructs containing a DNA sequence encoding PGX wherein the protein encoding sequence is operably linked to regulatory regions capable of directing the expression of said DNA sequence in a suitable host organism.

The invention further discloses DNA vectors containing the DNA constructs encoding PGX.

Transformed microbial host cells containing the vectors are disclosed and the host cells are shown to produce exo-polygalacturonase under suitable growth conditions.

The present invention discloses a method for obtaining expression of exo-polygalacturonase comprising, culturing the microbial host under conditions wherein the cloned gene is expressed. A method is also disclosed for obtaining exo-polygalacturonase comprising the steps of culturing the transformed microbial host under conditions giving rise to the expression of the DNA sequence of and recovering the polypeptide.

The invention also discloses the use of a polypeptide obtained through the expression of the cloned pgaX gene to degrade pectin. It is shown in the present invention that the pectin can be specifically treated using the exo-polygalacturonase. It is disclosed that this enzyme reduces

the calcium sensitivity without at the same time reducing the molecular weight of pectin.

Furthermore, the invention provides purified and isolated expression and transcription regulatory regions as found in the 5'non-coding sequences of the Aspergillus tubigensis pgaX gene.

The present invention describes a method for obtaining a gene encoding an Aspergillus exo-polygalacturonase. The method can be summmarized as follows.

An Aspergillus strain, which may be selected from the group comprising A. niger, A. awamori and A. tubigensis is grown under conditions wherein the carbon source is limited and wherein pectin or fragments thereof are offered as substrate. These growth conditions give rise to the production of pectin degrading enzymes.

Following sufficient growth time allowing for the accumulation of the different enzymes the cells are harvested and the enzymes are isolated from the medium. After suitable identification the exo-polygalacturonase is purified and sequenced. This sequencing may be performed on the complete protein molecule it may however also be performed on fragments obtained after separation of the peptides following peptidase digestion of the complete protein.

Once the amino acid sequence or parts thereof are determined, suitable sequences are selected for choosing DNA probes. The probes are used against a genomic library of the desired Aspergillus strain. The hybridizing clones are selected and the DNA fragments contained therein are sequenced. The sequence is further completed by adding the rest of the sequence.

Suitable expression regulating sequences are then cloned upstream from the coding region. This depends on the preferred expression host cell. Expression is thus made constitutive.

The cloned gene is expressed and the medium can be used as such in the degradation of pectin. Preferably, the protein is isolated and used in the desired process.

Alternative ways for cloning, detecting, screening and expressing the gene are known and are expected to give similar results. Among these methods are methods using expression cloning combined with immunological detection, another method would be the use of PCR to amplify the DNA encoding the exo-polygalacturonase gene, this presupposes however that at least a part of the gene sequence is known.

Reaction of the exo-polygalacturonase of the present invention with pectin or pectin-like substrates gives rise to the production of galacturonic acids in monomeric or dimeric form. Galacturonic acid can for example be used in the synthesis of ascorbic acid. Unlike known processes the use of exo-polygalacturonase in pure form gives rise to less by-product formation.

Furthermore, the exo-polygalacturonase of the present invention can be used in combination with pectinesterase, pectinase and other pectin degrading enzymes. It is further shown in the examples that exo-polygalacturonase can be used to treat pectin during this treatment the viscosity of the pectin is reduced without reducing the molecular weight of this polymer.

Experimental

Throughout this description the following strains and vectors are employed:

Strains:

E. coli JM101 (Yanisch-Perron et al., 1985):
thi, Δ(lac-supE, pro AB), [F', traD36, pro AB, lacI^{q}ZΔM15]

E. coli LE 392 (Murray, 1977):
F$^-$, hsdR574 (r_k^+,m_k^+), sup44, supF58, lacY1, or Δ(lac1ZY)6, galK2, galT22, metB1, trpR55, λ$^-$

Aspergillus niger N402: cspA1
Aspergillus niger N593: cspA1, pyrA6
Aspergillus tubigensis NW756
Aspergillus tubigensis NW218: pyrA22
Aspergillus nidulans G191: pabaA1, pyrG89, fwA1, uaY9

Vectors:
pEMBL18/pEMBL19 (Dente et al., 1983)

Example 1

Purification and characterization of Aspergillus tubigensis exo-polygalacturonase PGX

Example 1.1

Purification of Aspergillus tubigensis exo-polygalacturonase PGX

A culture filtrate (3000 ml) was obtained by the culturing of Aspergillus niger strain NW756 (later

reclassified as more likely belonging to the species A. tubigensis (Kusters-van Someren et al. (1991)) in a medium containing (per liter) 7.5 g NH_4NO_3, 1.5 g KH_2PO4, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 2 g yeast extract, trace elements according to Vishniac and Santer (1957) and 10 g degraded polygalacturonic acid as carbon source, pH 6.0.

Polygalacturonic acid was degraded as follows : 40 g polygalacturonic acid (United States Biochemical Corp.) was dissolved in two liters 20 mM sodium acetate buffer pH 4.8 and incubated with 4000 Units of purified A. niger endopolygalacturonase II, of which the purification is described by Kester and Visser (1990), for 24 hours at 30°C.

The pH of the medium was adjusted to 6.0 following inoculation at 10^6 spores/ml and incubation at 30°C in an orbital shaker at 250 rpm for 55 hours. The culture fluid was passed through cheese cloth and the filtrate was brought to 10 mM sodium acetate, adjusted to pH 4.0 and loaded on a crosslinked alginate column (100 ml) in 20 mM sodium acetate buffer pH 4.0.

The non-bound fraction which contained a mixture of residual endo-PG activity and exo-polygalacturonase activity was concentrated by batch wise adsorption on DEAE Sephadex A-50 (200 g wet weight) at pH 6.0 followed by pulse elution with 1 M NaCl in 20 mM sodium phosphate buffer pH 6.0 and dialysis against the same buffer without the salt. The dialysate was loaded on a DEAE-Sepharose Fast Flow column (2.5 x 10 cm) equilibrated in 10 mM bisTris/HCl buffer pH 5.5.

After elution with a 800 ml linear sodium chloride gradient (0-0.5 M), fractions were assayed for polygalacturonase activity and screened for exo-poly-galacturonase activity by a TLC method. This method consists of incubation of samples of the column fractions with 1 % (w/v) polygalacturonic acid in 0.1 M sodium acetate buffer pH 4.2 at 30°C followed by analysis of the reaction products by thin layer chromatography as described by Kester and Visser (1990).

Exo-polygalacturonase positive fractions were pooled, dialyzed against 20 mM sodium acetate buffer pH 4.0 and loaded on a S-Sepharose Fast Flow column (2.5 x 7 cm) equilibrated in the same buffer. Elution was done with a 600 ml linear sodium chloride gradient (0-0.5 M).

Final purification was accomplished by repeated chromatography on a MONO Q column (Pharmacia, Uppsala, Sweden) in 20 mM piperazine/HCl buffer pH 6.0. Exo-polygalacturonase containing fractions were diluted five times with water loaded on the column and eluted with a 40 ml linear sodium chloride gradient (0-0.2 M) at a flow rate of 2 ml/minute. A summary of the specific activity and recovery of the enzyme during purification is given in Table I.

Table I. Purification of exo-polygalacturonase PGX from A. tubigensis NW756

Step	Total Volume (ml)	Total activity (units)	Specific protein (mg)	activity (units/mg)	Yield (%)
Culture fluid	3000	5230	300	17.4	100
Crosslinked alginate	210	3100	212	14.6	59
DEAE-Sepharose	90	846	98	8.6	16
S-Sepharose	72	188	8.8	21.4	3.6
MONO Q	10.8	121	3.1	39.0	2.3

Polygalacturonase activity was determined by measuring the release of reducing sugars in a reaction mixture containing 0.25 % (w/v) polygalacturonic acid in 0.1 M sodium acetate buffer pH 4.2 at 30°C. Reducing end groups were determined by the neocuproine method described by Stephens et al. (1974). One activity unit was defined as the amount of enzyme which produces one μmole of reducing sugars per minute.

The apparent molecular mass of exo-polygalacturonase PGX, as determined by SDS-PAGE on a 10 % gel, was 78 kDa. After deglycosylation of the enzyme with N-glycanase (Genzyme, Cambridge, Mass.) according to the supplier instructions the molecular weight was reduced to 52 kDa.

Isoelectric focussing resulted in five distinct bands, all showing exo-polygalacturonase activity, in the pH range 3.7-4.4.

Example 1.2

Amino acid sequence determination of the N-termini of cyanogen bromide released peptides of exo-polygalacturonase PGX

Approximately 150 µg of exo-polygalacturonase PGX, purified as described in Example 1.1, was extensively dialysed against distilled water, lyophylized and dissolved in 100 µl 70 % (v/v) formic acid-water mixture. To this protein solution 500 µg cyanogen bromide (2500 molar excess), dissolved in the formic acid-water mixture, was added. The reaction was performed for 24 hours at room temperature after which the reaction mixture was diluted 10 fold with water following lyophylization.

Approximately one half of the reaction mixture was subjected to electrophoresis on a 15 % polyacrylamide gel, followed by blotting onto Immobilon-P membrane (Millipore) according to the method described by Matsudaira (1987). Membrane slices containing fragments with a molecular mass of 30 kD (CB 1), 27.5 kD (CB 2) and 23.5 kD (CB 3) respectively were used in gas-phase sequencing (Applied Biosystems model 470A protein sequencing, SON, Leiden) according to the program described by Amons (1987).

The following N-terminal amino acid sequences were determined:

CB 1 (L2278):

1 5 10
Ala-(Arg)-Ile-Lys-Val-(Trp)-Pro-Gly-Thr-Pro-Ser-Ala-(Leu)-?-(Ala)

(Formula 1) = (SEQ ID NO: 1)

CB 2 (L1742):

1 5 10
(Gly)-Ile-Ile-Gly-Leu-Asn-Gly-Gly-Thr-Ile-Gly-Pro-Leu-Lys-

15 20
Leu-(Arg)-Tyr-Ser-Pro-Gln

(Formula 2) = (SEQ ID NO: 2)

CB 3 (L1743):

1 5 10
Phe-Ser-(Leu)-Ser-?-Glu-Ala-Ala-(Thr)-Gly-Pro-Lys-Lys-(Pro)-Phe-?-? Leu-(Leu)

(Formula 3) = (SEQ ID NO: 3)

Example 2

Screening of the genomic library of Aspergillus niger DS16813 (CBS 323.90; later classified as A. tubigensis) for the exo-polygalacturonase gene (pgaX) and isolation of the gene

Example 2.1

Synthesis of a pgaX specific PCR fragment using oligonucleotide mixes derived from PGX amino acid sequences.

The amino acid sequences determined in Example 1.2 (Formulas 1, 2 and 3) were used to synthesize oligonucleotide mixes corresponding to the coding and the non-coding DNA strands. The oligonucleotides are synthesized by the phosphoramidite method, using an Applied Biosystems oligonucleotide synthesizer.

The oligonucleotide mixes (Figure 1) were used in PCR with chromosomal DNA of A. tubigensis NW756 as template. All possible combinations of two oligonucleotide mixtures (100 pmole each) were used in reactions also containing 0.5 µg chromosomal A. tubigensis NW756 DNA, 200 µM dNTPs, Taq buffer (Sphaero-Q) and 0.5 U Taq polymerase (SuperTaq, Sphaero-Q). Conditions for PCR were 25 cycles of melting at 94°C (1 minute), annealing at 40°C (1 minute) and polymerization at 72°C (2 minutes).

The reaction products were analyzed by electrophoresis using a 2% TAE-agarose gel. Only the combination of oligonucleotide mixture 3059 and 3062 resulted in a reaction product, which was approximately 600 bp in length. This PCR fragment was purified using a the Magic PCR Preps DNA purification system (Promega) following the instructions of the manufacturer.

Example 2.2

^{32}P labelling of DNA fragments

After purification, the DNA concentration of the PCR fragment described in Example 2.1 was determined. 50 ng was labelled by random priming using the Prime-a-Gene kit from Promega. To remove the unincorporated α-32P-dATP from the mixture, the volume was increased to 100 µl with TE buffer, after which the unincorporated α-32P-dATP was removed by fractionation on a Sephadex G50 column. Fractions containing the radioactively labelled DNA were denatured by incubation for three minutes at 100°C, and kept single stranded by rapidly chilling on ice, before addition to a hybridization buffer containing 6 x SSC, 5 x Denhardt's solution, 0.1% sodium pyrophosphate and 100 µg/ml heat-denatured herring sperm DNA.

This probe was used in the screening of the genomic library (Example 2.3) and in Southern blot analysis (Examples 2.4, 2.5 and 5.1).

Example 2.3

Screening of the Aspergillus tubigensis genomic library for the pgaX gene

To screen for the pgaX gene in an Aspergillus tubigensis genomic library, constructed as described in case EP 0 463 706 A1, Example 2, 5.10^3 pfu per plate were plated in LM top agarose containing 0.7% agarose on 8 85 mm diameter LM (1.2% agar) plates as described (Maniatis et al., 1982, pp. 64). E. coli LE392 were used as plating bacteria.

After incubation of the plates overnight at 37°C, two replicas of each plate were made on nitrocellulose filters (Schleicher and Schüll BA85) as described in Maniatis et al. (1982, pp. 320-321).

After baking the filters for 2 hours at 80°C, the filters were prehybridized at 65°C for two hours in a prehybridization buffer containing 6xSSC, 0.5 % SDS, 10xDenhardt's solution and 100 µg/ml heat denatured herring

sperm DNA (Boehringer). The heat-denatured probe was added and the filters were hybridized for 18 hours at 65°C.

After hybridization, the filters were first washed in 2 x SSC, 0.5% SDS, and then in 0.2 x SSC, 0.5% SDS at 65°C. The air dried filters were taped onto a sheet of Whatman 3MM paper, keying marks were made with radioactive ink and the Whatman paper and filters were covered with Saran Wrap™. Hybridizing plaques were identified by exposure of Kodak XAR X-ray film for 18 hours at -70°C using an intensifying screen.

Twelve plaques hybridizing with the PCR probe, appearing in duplicate on the replica filters, were identified: $\lambda_{pga}1$ to $\lambda_{pga}12$. Each positive plaque was removed from the plate using a Pasteur pipette and the phages were eluted from the agar plug in 1 ml of SM buffer containing 20 µl chloroform, as described in Maniatis et al. (1982, pp. 64). The phages obtained were purified by repeating the procedure described above using filter replicas from plates containing 50-100 plaques of the isolated phages.

After purification, the phages were propagated by plating $5x10^3$ phages on LM medium. After incubation overnight at 37°C, confluent plates were obtained, from which the phages were eluted by adding 5 ml SM buffer and storing the plate for 4 hours at 4°C with intermittent shaking. After collection of the supernatant, the bacteria are removed from the solution by centrifugation at 4,000 x g for 10 minutes at 4°C. Chloroform (0.3%) was added to the supernatant and the number of pfu was determined. These phage stocks contained approximately 10^{10} pfu/ml.

Example 2.4
Isolation of DNA from bacteriophage lambda

Eight of the isolated phages λ_{pgaX} 1, 2, 4, 5, 6, 7, 8 and 11 were propagated as described in Example 2.2 using five plates for each of the phages. The phages were precipitated

from the thus-obtained supernatant (25 ml) by addition of an equal volume of a solution containing 20% PEG-6000 (w/v) and 2 M NaCl, followed by thorough mixing and incubation on ice for 60 minutes.

The precipitated phages were collected by centrifugation at 14,000 x g at 4°C for 20 minutes. The supernatant was removed by aspiration. The phages were carefully resuspended in 4 ml SM buffer and extracted once with chloroform.

Prior to extracting the DNA from the phage particles, DNA and RNA originating from the lysed bacteria were removed by incubation of the phage suspension with DNase I and RNase A (both 100 µg/ml) for 30 minutes at 37°C. The phage DNA was subsequently released from the phages by extraction with an equal volume of phenol/chloroform (1:1). After separation of the phases by centrifugation using a Sorvall centrifuge (14,000 x g, 10 minutes), the aqueous phase was extracted once with an equal volume chloroform. The phases were separated by centrifugation (Sorvall centrifuge, 14,000 x g, 10 minutes) after which the DNA was precipitated from the aqueous phase by the addition of 0.1 volume 3 M sodiumacetate and 2 volumes ethanol. After mixing, the DNA was recovered by centrifugation in a Sorvall centrifuge for 10 minutes at 4°C (14,000 x g).

The supernatant was removed by aspiration after which the DNA was resuspended in 400 µl TE buffer. The DNA was precipitated once again from this solution by the addition of 0.1 volume 3 M sodium acetate and 2 volumes ethanol. The DNA was collected by centrifugation for 10 minutes at 4°C (14,000 x g). The supernatant was removed by aspiration, the remaining pellet was briefly dried under vacuum, after which the DNA was resuspended in 125 µl TE buffer containing 0.1 µg/ml RNase A. This purification procedure resulted in the isolation of approximately 10-50 µg DNA from each phage.

Example 2.5

Restriction analysis of pgaX containing phages

The isolated DNA of phages λ_{pgaX} 1, 2, 4, 5, 6, 7, 8 and 11 was analyzed by Southern analysis using the following restriction enzymes: PvuII and HincII. The DNA was digested for 3 hours at 37°C in a reaction mixture composed of the following solutions; 3 µl (≈ 1 µg) DNA solution; 10 µl of the appropriate 10 x React buffer (BRL); 20 U restriction enzyme (BRL) and sterile distilled water to give a final volume of 100 µl.

After digestion the DNA was precipitated by the addition of 0.1 volume 3 M NaAc and 2 volumes ethanol. The DNA was collected by centrifugation for 10 minutes at room temperature (14,000 x g). The supernatant was removed by aspiration, the remaining pellet was briefly dried under vacuum and resuspended in sterile distilled water.

After addition of 4 µl DNA loading buffer the samples were incubated for 10 minutes at 65°C and rapidly cooled on ice. Samples were loaded on a 0.6% agarose gel in TAE buffer. The DNA fragments were separated by electrophoresis at 25 V for 15-18 hours.

After electrophoresis the DNA was transferred to nylon membrane (Gene Bind 45, Pharmacia LKB) by Southern blotting as described in Maniatis et al. (1982) and subsequently prehybridized and hybridized using the labeled PCR fragment as described in Example 2.1 and hybridization conditions as described in Example 2.2. The hybridization pattern was obtained by exposure of Kodak XAR-5 X-ray film for 18 hours at -70°C using an intensifying screen.

From the results obtained it was concluded that the DNA of all isolated clones hybridize with the PCR fragment. Two clones appeared to be identical and contained a very large hybridizing PvuII fragment. No hybridizing HincII fragment was detected, suggesting that this fragment was too small and had run off the gel. In six clones fragments originating from the same genomic region were found. In a more extensive

Southern analysis, using the enzymes XhoI, PvuII, PstI, BglII, SalI, EcoRI and BamHI a partial restriction map of this genomic region was constructed. From this experiment it was concluded that a 5.8 kb XhoI fragment contains the A. tubigensis pgaX gene.

Example 2.6
Subcloning of the A. tubigensis pgaX gene

From phage $\lambda_{pgaX}7$ the 5.8 kb XhoI fragment was isolated by digesting the phage DNA with XhoI and separation of the fragments as described in Example 2.4. The fragment was cut from the agarose gel, after which it was recovered from the agarose using GeneClean (Bio101) as described by the manufacturer. The DNA was dissolved in 10 µl sterile water and the concentration was determined by agarose electrophoresis, using lambda DNA with a known concentration as a reference and ethidiumbromide staining to detect the DNA.

The fragment obtained was ligated in the vector pEMBL19 digested with SalI and dephosphorylated with alkaline phosphatase prepared as follows; 1 µl (1 µg/µl) pEMBL19 was mixed with 2 µl 10 x React 10 (BRL), 1 µl (1 U/µl) SalI and 16 µl sterile distilled water. The DNA was digested for 1 hour at 37°C, after which 0.5 µl alkaline phosphatase (1 U/µl) (Pharmacia LKB) was added followed by further incubation at 37°C for another 10 minutes.

The 5.8 kb XhoI fragment was ligated in the vector resulting in the plasmid pIM362, by the following procedure: 100 ng pEMBL19 fragment was mixed with 100 ng 5.8 kb XhoI fragment and 4 µl 5 * ligation buffer (BRL) and 1 µl (1.2 U/µl) DNA ligase (BRL) was added to this mixture in a final volume of 20 µl. After incubation for 16 hours at 4°C, 10 µl of the mixture was used to transform E. coli JM101 competent cells, prepared by the CM1, CM2 method as described in the Pharmacia Manual for the M13 cloning/sequencing system. One of the resulting colonies was grown overnight in LB medium

containing 100 µg/ml ampicillin. From the culture plasmid DNA was isolated by the alkaline lysis method as described by Maniatis et al. (1982, pp. 368-369), which was used in restriction analysis, as described in Example 2.4 to check whether it harboured the desired plasmid. Plasmid DNA was isolated on a large scale from 500 ml cultures E. coli JM101 containing the plasmid pIM362 grown in LB medium containing 100 µg/ml ampicillin (Maniatis et al., 1982, p.86) The plasmid was purified by CsCl density gradient centrifugation, phenolized, ethanol precipitated and dissolved in 400 µl TE. The yield was approximately 500 µg.

Similarly, a 4.0 kb PvuII fragment which is located within the 5.8 kb XhoI fragment, was isolated from phage 7, subcloned in pEMBL19 digested with SmaI, resulting in plasmid pIM361.

The plasmid pIM361 was further analyzed by restriction enzymes resulting in the restriction map shown in Figure 2.

Plasmids pIM361 and pIM362 containing the exo-PG gene have been deposited in E.coli JM101 at the Centraal Bureau voor de Schimmelcultures in Baarn, The Netherlands and have received the deposition number CBS 101.93 and CBS 102.93, respectively.

Example 3
Characterization of the Aspergillus tubigensis pgaX gene

Example 3.1
Sequence determination of the A. tubigensis pgaX gene

The sequence of the A. tubigensis pgaX gene was determined by subcloning fragments from pIM362 in pEMBL18/19, in combination with the use of specific oligonucleotides as primers in the sequencing reactions.

For nucleotide sequence analysis restriction fragments were isolated as described in Example 2.5 and then cloned in pEMBL18/19 vectors digested with the appropriate restriction enzymes, as described in Example 2.5. The nucleotide sequences were determined by the dideoxynucleotide chain-termination procedure (Sanger et al., 1977) using the Pharmacia T_7 DNA polymerase sequencing kit. Computer analysis was done using the PC/GENE program (Intelligenetics, Inc.: Madison WI). The sequence was determined for 98% in both orientations Sequence Listing). The sequence includes about 640 bp of the 5′ and 655 bp of the 3′ sequence.

Example 3.2
The A. tubigensis pgaX gene structure

There are probably 6 introns present in the gene. This is based on the appearance of in-frame stopcodons in all of the introns and on the presence of intron consensus sequences (Table II).

Table II. Intron sequences of the pgaX gene.

	position	length	5' site	lariat	3' site
Intron I	948-1004	57	GTGctT	ACTAAC	CAG
Intron II	1372-1423	52	GTGcGT	ACTGAC	TAG
Intron III	1483-1540	58	GTAaGT	tCTAAC	CAG
Intron IV	1942-1991	50	GTtaGT	GCTAtC	TAG
Intron V	2094-2145	52	GTAaGT	gCTGAC	TAG
Intron VI	2221-2273	53	GTGaGT	ACTAAC	CAG
consensus			GTPuNGT	PuCTPuAC	PyAG

The pgaX gene encodes a secreted protein of 47.1 kD and an iso-electric point of 4.11. The calculated molecular weight is lower than the one determined by SDS-PAGE (78 kD), even after deglycosylation (52 kD) (there are 12 possible N-glycosylation sites in PGX). Discrepancies between calculated and determined molecular weights have also been found for other proteins. All three CNBr fragments are found in the sequence. L1743 appears to represent the N-terminal amino acid sequence of the mature exo-PG protein: no methionine is found immediately preceding the phenylalanine (F), which would have been expected if it were a true CNBr fragment. Instead a sequence with the characteristics of a signal sequence is found. According to the computer program PC-GENE the preferred signal sequence splice site is between serine (S 24) and arginine (R 26), however, since these amino acids are found in the mature protein, this is not the case. The splice site between the alanine (A 22) and phenylalanine (F 23) is second best.

Example 4

Expression of the pgaX gene in Aspergillus niger N593, Aspergillus tubigensis NW218 and Aspergillus nidulans G191

Example 4.1

Introduction of the pgaX gene into Aspergillus niger N593, Aspergillus tubigensis NW218 and Aspergillus nidulans G191 by cotransformation

The plasmids pIM361 and pIM362, obtained in Example 2.5, were introduced in A.niger, A. tubigensis and A. nidulans by cotransformation of A. niger N593, A. tubigensis NW218 and A. nidulans G191 using the A. niger pyrA gene as a selective marker on the plasmid pGW635 (Goosen et al., 1989) and the plasmids pIM361 and pIM362 as the cotransforming plasmids in separate experiments.

Protoplasts were prepared from mycelium by growing A. niger N593 and A. tubigensis NW218 on minimal medium supplemented with 0.5% yeast extract, 0.2% casamino acids, 50 mM glucose and 10 mM uridine for 20 hours at 30°C. For the preparation of protoplasts A. nidulans G191 was grown at 37°C on the same medium supplemented with 1.35 mg/l p-aminobenzoate. The preparation of protoplasts and the transformation procedures were performed as described by Goosen et al., 1987. The resulting pyr^+ transformants were then analyzed for the expression of the pgaX gene by Western blot analysis.

Example 4.2

Screening of transformants for the expression of the pgaX gene

The transformants obtained in Example 4.1 were analyzed for the formation of the pgaX gene product, the PGX protein. 10 pIM361 and 10 pIM362 A. niger transformants, 5 pIM361 and

5 pIM362 A. tubigensis transformants and 10 pIM361 and 10 pIM362 A. nidulans transformants were selected and grown for 24 hours on medium containing per liter 10 g PGII-degraded polygalacturonic acid, 7.5 g NH_4NO_3, 0.5 g KCl, 0.5 g $MgSO4.7H_2O$, 1.5 g $KH2PO_4$, 0.2% yeast extract, 1 ml/l Vishniac solution and 1.35 mg/l p-aminobenzoate for A. nidulans only (pH 6.0).

After growth (24 hours after inoculation) the mycelium was removed by filtration and the proteins in the culture filtrates were analyzed by SDS-polyacrylamide gel electrophoresis, using a gel containing 10% acrylamide.

The PGX protein was detected on nitrocellulose after electroblotting and incubation with polyclonal antibodies raised against the PGX protein purified as described in Example 1.1. The antibody bound, was detected after incubation with goat-anti-mouse antibody conjugated to alkaline phosphatase, according to the Biorad instruction manual.

Table III. Transformants which overproduce PGX.

		t=24
A. tubigensis	(pIM361)4	++
	(pIM361)6	+++
	(pIM362)7	+
A. nidulans	(pIM361)6	++
	(pIM361)9	+++
	(pIM362)2	++

The amount of PGX produced as judged by Western analysis is indicated.

+++: high overproduction as compared to the corresponding wild type strain.

+ : low overproduction as compared to the corresponding wild type strain.

Table III shows the results of the Western analysis. Only A. tubigensis and A. nidulans transformants readily overproduced the PGX protein as detected by this procedure. The protein was secreted into the medium. Of the A. tubigensis transformants analyzed one was selected for giving the highest yields of the PGX protein, transformant A. tubigensis NW218(pIM361)6.

In a second experiment A. tubigensis NW218 was transformed with 1 µg of pGW613 which contains the A. niger pyrA gene and 40 µg pIM361. The highest producer found in this experiment was NW218 (pIM361)22.

Example 4.3

Overexpression of PGX in A. tubigensis transformants and purification and characterization of PGX

PGX was purified from A. tubigensis NW756. NW756 was grown for 48 h at 30°C in medium containing 7.5 g ammonium nitrate. 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 2 g yeast extract, trace elements and 1% PGII-degraded polygalacturonic acid, pH 6.0. The culture fluid was harvested by filtration and the pH (initial value 6.8) was adjusted to 6.0. The culture filtrate was stirred for one hour with DEAE-Sephadex A50 (300 g, wet weight), equilibrated in 10 mM sodium phosphate buffer pH 6.0. The ion-exchanger was collected, poured into a column, and bound protein was eluted by a pulse of 1 M NaCl in buffer. After dialysis against 20 mM sodium acetate buffer pH 4.2 the solution was loaded on a cross-linked alginate column (0=5 cm, h=8 cm) in the same buffer followed by elution with two pulses of respectively 0.3 and 1 M NaCl in buffer. Part of the exo-PG activity did bind to the crosslinking alginate column and was eluted by the 0.3 M NaCl pulse (pool I). The non-bound fraction was dialyzed against 20 mM sodium citrate buffer pH 3.5 and loaded on a S-Sepharose Fast Flow column (0=2.5 cm, h=25 cm) equilibrated in the same buffer followed by elution by a 600 ml NaCl gradient (0-0.6 M) (pools I, II, III). These pools all contained exo-PG activity and all also contained endo-PG activity except pool I of the S-Sepharose column.

All four pools were concentrated up to 5 ml by adsorption on a small (0=1.5 cm, h=5 cm) DEAE-Sepharose Fast Flow column at pH 6.0 followed by pulse elution with 1 M NaCl in 10 mM sodium phosphate buffer pH 6.0. The final purification was done by GPC on a Sephacryl-S 200 column (0=2.5 cm, h=90 cm) in 0.1 M sodium acetate buffer pH 4.8.

No endo-PG and PL activity could be detected in these final samples.

	volume	U/ml	mg/ml	S.A.
crl. gen. pool I/GPC/conc.	9.8	19.4	0.54	36
S-Seph. pool II/GPC	22.7	1.28	0.017	75
S-Seph. pool III/GPC/conc.	6.5	14.4	0.31	46.5

S.A. = specific activity

Purification of PGX from transformant NW218 (pIM361)22.

Cultivation was done for 50 h at 30°C in minimal medium with ammonium nitrate as nitrogen source and 1% PGII-degraded polygalacturonic acid as carbon source. After 24 and 50 h only one major protein could be detected in the culture fluid as judged by Coomassie Brilliant Blue-staining following SDS-polyacylamide gel electrophoresis. Total protein in the culture filtrate was concentrated by batch wise adsorption on DEAE A50 at pH 6.0 followed by pulse elution with 1 M NaCl in 20 mM sodium phosphate buffer pH 6.0.

After extensive dialysis against 20 mM sodium acetate buffer pH 3.6 dialysate was loaded onto a S-Sepharose Fast Flow column (2.5 * 20 cm) equilibrated in the same buffer. Elution was done with a linear NaCl gradient (800 ml, 0 - 0.5 M).

Fractions were screened by SDS-PAGE and based on these results two pools were formed which were used for further analysis.

	U/ml	mg/ml	U/mg	mg	total U
Pool I	39.5	0.87	45.4	61	2.765
Pool II	400	5.1	78	790	12.900

Based on these results the production level of PGX in this transformant (approx. 180 mg/l) is 80 fold higher than for the wild type strain NW756.

After one purification step the preparation is not completely pure, but no pectin lyase and no endo-PG activity

could be detected. Pool I contains a low level of pectinesterase activity.

Example 4.4

Deregulated expression of the A. tubigensis pgaX gene

To make a gene fusion of the A. niger N400 pki promoter and the A. tubigensis DS16813 pgaX gene, first a PCR fragment was made using a primer which contains the pki SmaI site and another primer containing the A. niger pki ATG and the first 12 nucleotides of the pgaX gene (pgaX sequences are shown in small letters):

pki SmaI

5′ TTTTCCTTCCCGGGCAC 3′
sense
SEQ ID NO: 6

pki-pgaX

3′ GTTAGTAGGCAGTTCTACtctgagtgcgtg 5′
antisense
SEQ ID NO: 7

Similarly, a PCR fragment was made using an oligo complementary to the pki-pgaX primer described above and another primer containing a NcoI site 165 bp upstream in the pgaX coding region:

pki-pgaX

5′ CAATCATCCGTCAAGATGagactcacgcac 3′
sense
SEQ ID NO: 8

pgaX NcoI

3′ cggtacctctaccgtga 5′
antisense
SEQ ID NO: 9

Subsequently, both PCR fragments were used in a third PCR experiment with only the pki SmaI primer and the pgaX NcoI primer. This resulted in a fragment of approx. 300 bp which was digested with SmaI and NcoI to obtain the desired sticky ends and the fragment was then further purified.

pIM361 was digested with BamHI which cuts in the pEMBL polylinker. A BamHI fragment containing the pki promoter and

the A. niger pelB gene was cloned into this plasmid. Digestion with NcoI and SmaI cut out the pelB gene as well as the 5′ end of the pgaX gene and the 3′ end of the pki promoter. Ligation of the SmaI-NcoI PCR fragment described above resulted in pIM365 which thus contains the A. niger pki promoter fused to the A. tubigensis pgaX gene. The construct was checked by sequencing, using the commercial universal and reverse primers as well as the pki-SmaI and pgaX-NcoI primers to sequence the PCR fragment.

A. tubigensis NW218 was transformed with the A. niger pyrA gene (pGW635, 1 μg) and pIM365 (40 μg) as cotransforming plasmid. Only two PGX overproducing transformants have been found, NW218 (pIM365) number 52 and 59, of which 59 has the highest production level. The copynumber of pgaX in this transformant has not yet been established.

Example 5

Screening for genes related to the pgaX gene in Aspergillus niger, Aspergillus tubigensis and Aspergillus nidulans

Example 5.1

Genomic hybridization of A. niger, A. tubigensis and A. nidulans DNA

High molecular weight DNA isolated from A. niger N400, A. tubigensis NW756 and A. nidulans WG312 as described in the published european patent application EP 0 463 706, Example 2.1, was digested with EcoRI, BamHI and HindIII. The resulting fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose membrane as described by Maniatis et al. (1982, pp. 383-389).

The nitrocellulose membranes were prehybridized at 60°C for two hours in hybridization buffer (as described in Example 2.2, above). After prehybridization, the radioactively labelled fragment, described in Example 2.2,

was added to the hybridization buffer and the hybridization was continued for 18 hours. After hybridization, the filters were washed for 60 minutes at 60°C in 4 x SSC, 0.5% SDS followed by final washing using 2 x SSC, 0.5% SDS at 60°C.

After taping the membranes to Whatmann 3MM paper and properly marking with radioactively labelled ink, the filters were covered with Saran Wrap™ and autoradiographed for 72 hours at -70°C using Kodak XAR-5 X-Ray film and Kodak X-Omatic cassettes with regular intensifying screens.

The hybridization fragments found are summarized in Table IV.

Table IV. Hybridizing fragments and their lengths (kb) found in A. niger, A. tubigensis and A. nidulans genomic DNA using a fragment of the A. tubigensis pgaX gene as a probe.

	EcoRI	BamHI	HindIII
A. niger N400	13	9.0	2.5
		6.0	1.9
		4.4*	1.6*
A. tubigensis NW756	5.5	23[1]	2.5
		12[1]	
		9.0*	
A. nidulans WG312	4.4	1.3	3.4

* : strongest hybridizing fragment

[1] : partially digested

Example 6

Efffect of exo-polygalacturonase on calcium sensitivity and molecular weight of pectin

Methods

Exo-PG and Rohament PL were used in these experiments.

Exo-PG was isolated from the supernatant of an A. tubigensis culture. Exo-PG was stored frozen in 20mM sodium citrate buffer pH 3.5. Exo-PG had an activity of 14.4 U/ml and a concentration of 0.31 mg/ml. No lyase and endo-polygalacturonase activities could be detected in the exo-PG solution. The activity was measured on polygalacturonic acid at a concentration of 0.25% (w/v) in 0.1 M sodium acetate pH 4.2 at 30°C.

Rohament PL was obtained from Rohm GmbH (Darmstadt, Germany). The activity was 2500 pgu/ml measured according to internal Rohm standards.

Pectin used for the test system has the following characteristics:

degree of ester = 71.7 (by titrimetry),

molecular weight = 117 kD, as described below,

calcium sensitivity = 213, according to the test described below.

Molecular weight determination for pectin

Principle

Molecular weight is estimated by measuring the relative viscosity of a 0.1% pectin solution using Na-hexametaphosphate.

Apparatus

1. Ostwald capillary tube viscosimeters (no less than two) with 100 to 150 sec. outlet time for water (25°C). For instance Silber Brand # 111.
2. Transparent thermostated water bath, 25.0°C ± 0.3°C.

Reagents

1. Na-hexametaphosphate solution:
   a) 20.0 g Na-hexametaphosphate is dissolved in 1800 ml ion exchanged deaerated (boiled) water.
   b) pH is adjusted to 4.50 ± 0.05 with 1 M HCl.
   c) The solution is diluted with ion exchanged, deaerated (boiled) water until 2000 ml.

Procedure

1. The viscosimeters must be cleaned appropriately.
2. Outlet time for hexametaphosphate solution is measured (section: Measuring of outlet time) on the viscosimeters used every time a new hexameta-phosphate solution is prepared and for every new working day where pectin solutions are being measured.
   Immediately before measuring the necessary quantity of hexametaphosphate solution is filtered through a glass filter # 3.
3. The pectin sample system for molecular weight determination is made as follows:
   a) Acid wash the pectin as described in the method for determination of AGA and DE (Food Chemicals Codex, 3rd Edition, Washington D.C., 1981).
   b) Approx. 90 g hexametaphosphate solution is weighed in a tared beaker with magnet.
   c) 0.1000 g acid washed pectin is gradually added while stirring. Keep stirring until the pectin is completely dissolved.
   d) Weigh up to 100.0 g with hexametaphosphate solution.
   e) Filter through a glass filter x 3.
4. For every molecular weight determination the outlet time is measured (section: Measuring the outlet time) for the pectin/hexametaphosphate solution on two different viscosimeters.

5. Molecular weight is calculated (section: Calculation) separately for each viscosimeter using the latest

measured outlet time for hexametaphosphate solution on the viscosimeter in question.

6. Should the difference between two calculated molecular weights be less than 3500 the mean value is calculated. Round off the value to the nearest multiple of 1000 and that will be the result of the method.
7. Should the difference between the two calculated molecular weights be 3500 or more the viscosimeters should be cleaned and a new measuring of outlet time for hexametaphosphate solution should be performed.

Measuring the outlet time

1. The viscosimeter is rinsed twice with the sample.
2. Pour 5.00 ml of the sample in the viscosimeter and place it in the thermostated water bath at 25.0°C ± 0.3°C at least 15 minutes prior to measuring.
3. Time is measured on two outlets. Should the difference between the times be more than x seconds the measuring is repeated until you have three outlet times which differ no more than x seconds.
   x = 0.2 seconds on measuring hexametaphosphate solution
   x = 0.4 seconds on measuring samples
4. The outlet time which is needed for further calculations is the mean value of the above mentioned two or three identical or almost identical measuring results.

Calculation

The relative viscosity is calculated, as follows:

$$n_r = \frac{(t_o - \frac{K}{t_o})}{(t_h - \frac{K}{t_h})}$$

Where t_o and t_h are outlet times for pectin solution and hexametaphosphate solution, respectively.

The parameter K can with sufficient accuracy be fixed at 75 s^2 using Silber Brand No. 111 viscosimeter. Otherwise, K can be calculated as follows:

$$K = \frac{Q \times t_v^2}{Q + (0.226 \cdot L \cdot t_v)}$$

where Q = volume of viscosimeter bulb in cm^3, L = length of capillary tube in cm and t_v outlet time for water in seconds.

The molecular weight of pectin is calculated as follows:

$$M = \frac{(n_r^{1/P} - 1) \cdot P}{k \cdot C}$$

where P is fixed at 6 and k is fixed at $4.7 \cdot 10^{-5}$ $mol \cdot g^{-1}$; C is the weight percentage of pectin in the sample system - i.e. 0.1% with the numerical values inserted you will obtain:

$M = 1.277 \cdot 10^6 (n_r^{1/6} - 1)$ g/mol.

Literature

P.E Christensen (1954) and Smit and Bryant (1967)

Calcium sensitivity test

A calcium containing pectin solution is prepared under conditions avoiding the formation of local gels during the addition of the calcium salt to the pectin solution.

The calcium salt and pectin are mixed at low pH prohibiting the reaction between calcium and pectin. This reaction is initiated by increasing the pH by adding an acetate buffer to obtain the desired pH.

Reagents:

1 M HCl
1 M Acetate buffer, pH 4.75
250 mM Calcium chloride solution

A pectin solution of the desired concentration is prepared in distilled water, and pH adjusted to 1.5 with 1 M HCl.

145 g portions of this pectin solution are measured into viscosity glasses.

5 ml of the calcium chloride solution is added to the 145 g pectin solution to give a final concentration of 8.3 mM Ca.

With efficient stirring with a magnetic stirrer, 25 ml of the acetate buffer is added to the pectin solution to bring pH to 4.2.

The magnet is taken out, and the glass is left at room temperature (25°C) until the next day, when the viscosity is measured with a Brookfield viscosimeter.

The pectin used in the present experiments had calcium sensitivity = 213, according to this test.

Test for polygalacturonase activity

Two enzymes are tested in every experiment: exo-PG and Rohament PL.

(1) A 1.6% pectin solution is prepared, and pH is adjusted to 3.8 with dilute NH_3 solution or dilute HCl. The solution is kept at 38°C.

(2) Three dilutions of each enzyme (in 20 mM sodium citrate buffer, pH 3.5) are prepared. The concentrations are chosen in accordance with the expected activity of the enzymes so a considerable (but not exhaustive) reduction of the calcium sensitivity of the pectin is achieved when the test system is incubated with the enzyme dilutions as will be detailed in the following

text. Further, the concentrations of the three dilutions are in the relative proportions 1 : 2 : 4.

(3) The pectin solution is split in nine portions, 95 g each. Three of these are incubated with 5 ml of each dilution of the "unknown" enzyme. Three portions are incubated with the "standard" enzyme. The remaining three portions are incubated with 5 ml water, they are referred to as "blanks".

(4) All nine test systems are treated in the same way:

(a) incubation at 38°C for 20 hours

(b) addition of 100 ml diluted HCl, the concentration is chosen by experience so that the pH of the test system becomes 1.8 ± 0.2

(c) heating on water bath for 4 minutes at 85°C, measured from the time when 70°C was reached (this treatment should destroy the enzyme)

(d) cooling to 25°C on water bath.

(5) Each of the so treated pectin solutions is split into two portions: one of these is made into - and measured as - a test system for pectin molecular weight, while the other is made into a test system for calcium sensitivity.

The test system for molecular weight is 0.1% pectin in sodium hexametaphosphate solution, pH 4.5. The viscosity is measured in an Ostwald capillary and converted to (what is reported as) molecular weight by a numerical expression. Details are given above

The test system for calcium sensitivity is 0.6% pectin in sodium acetate buffer with added Ca^{++}, pH 4.4. The viscosity is measured by means of a Brookfield viscosimeter. Details may be found above.

Results

The results are reported in a diagram with MW-change (in kD, relative to the average of the blanks) as the x-axis and calcium sensitivity (in cp) as the y-axis. An enzyme showing desired performance, i.e. a low calcium sensitivity

is achieved with almost no loss in molecular weight, will have data points in the lower right corner of the diagram.

Results of the two independent experiments are shown as Figures 3 and 4. The dosages of enzyme in mg per g pectin are printed next to the data points.

The Figures presented lead to the conclusion that:

(1) The exo-PG preparations can reduce the calcium sensitivity of the pectin that was used in the test system

(2) In both experiments, data points for the exo-PGs tend to be to the lower right side of the data points for the standard (Rohament PL) in the diagrams, Figures 3 and 4. This suggests that for this particular purpose an exo-PG is more desirable in its way of action than Rohament PL.

The above data suggest that a great part of the calcium sensitivity of pectin is due to blocks situated at the non-reducing ends of some of the pectin molecules.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Gist-brocades
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(ii) TITLE OF INVENTION: Cloning and expression of the exo-polygalacturonase gene from Aspergillus

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: L2278

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Xaa Ile Lys Val Xaa Pro Gly Thr Pro Ser Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: L1742

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ile Ile Gly Leu Asn Gly Gly Thr Ile Gly Pro Leu Lys Leu Xaa Tyr
1 5 10 15

Ser Pro Gln

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: L1743

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Phe Ser Xaa Ser Xaa Glu Ala Ala Xaa Gly Pro Lys Lys Xaa Phe Xaa
1 5 10 15

Xaa Leu

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2974 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus tubigensis
(B) STRAIN: NW756

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 640..946

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 947..1003

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1004..1370

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1371..1422

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1423..1481

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1482..1539

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1540..1940

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1941..1990

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1991..2092

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2093..2144

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 2145..2219

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2220..2272

(ix) FEATURE:
(A) NAME/KEY: exon

(B) LOCATION: 2273..2320

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(640..946, 1004..1370, 1423..1481, 1540..1940, 1991..2092, 2145..2219, 2273..2320)
(D) OTHER INFORMATION: /codon_start= 640
/product= "exo-polygalacturonase precursor"
/gene= "pgaX"

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 640..705

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 706..2320
(C) IDENTIFICATION METHOD: experimental
(D) OTHER INFORMATION: /product= "exo-polygalacturonase mature enzyme"
/evidence= EXPERIMENTAL

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
GACTCTAGAG GATCCCCCTG TCGGCCTTTT AAATGGCTTC CTAGTGGTGG TGTATTGTCC      60

ACGAGGCTTG TACCACACGG AGTTTGCACT GACCTGCACT TACCGCAAGT TTCGGGAATT     120

GACCTGCGCG ACAATATGAT CTAACGGGGA TTAAAGAATA ACCGGGTATT GCTCTCTCAA     180

TTAAAATGAA ATCATGATGC AGAAGATGGC TGGGGTAAGT TCCCTGCCAA TTCCCCAGAA     240

ACACGTTTTT CCCCCAATCG GATAATCGTC TCCGACAAGC CCTCCTATCA CATCGTCTCT     300

CGTTGCTCCT CCACGCAATC CCCCACCACC ACATTCTCCA GCAGCTAACT GTCTCCAAGT     360

GCCCCTGTTG ATTACAGGAG CAAGCCACAA GCTTAGCATA GCTCCACAGC CGTGGTGTGC     420

TCACTCCGTT TGGTCGATTT TGTTTCCCCG GATATGGAGT CCAGCGAACT CCCCAACTCG     480

GAGACATGGG TGGTGTGGTC AGTGTGAGCA AGACTGCGGG GGAGCAATGT CGACTCAGTG     540

CAATGGATAT AAATGCCAGT GGCGATCGTC GTTGCTCTGC GGTAATCCTG CCCAAGCTCT     600

GTCCGTGAAC CCAGACTTGT CTCCTGCCAA TTACACACA ATG AGA CTC ACG CAC        654
                                              Met Arg Leu Thr His
                                              -22  -20

GTT CTC TCG CAC ACG CTT GGC CTT CTT GCG CTA GGG GCC ACA GCA GAG      702
Val Leu Ser His Thr Leu Gly Leu Leu Ala Leu Gly Ala Thr Ala Glu
-15                  -10                  -5

GCC TTC TCC CGA TCC AGA GAA GCT GCC TGC GGC CCA AAA AAG CCT TTC      750
Ala Phe Ser Arg Ser Arg Glu Ala Ala Cys Gly Pro Lys Lys Pro Phe
 1               5                  10                  15
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CGG CCT CTA CCT ACA AGC CAG AGC AGG GAC AAG ACC TGC CAT GTC CGC          798
Arg Pro Leu Pro Thr Ser Gln Ser Arg Asp Lys Thr Cys His Val Arg
                20                  25                  30

AGC CAT GGA GAT GGC ACT GAC GAC TCT GAT TAC ATT CTC TCC GCA TTG          846
Ser His Gly Asp Gly Thr Asp Asp Ser Asp Tyr Ile Leu Ser Ala Leu
            35                  40                  45

AAC CAA TGT AAC CAC GGT GGA AAG GTA GTT TTT GAT GAG GAC AAG GAA          894
Asn Gln Cys Asn His Gly Gly Lys Val Val Phe Asp Glu Asp Lys Glu
        50                  55                  60

TAC ATT ATC GGG ACG GCA CTG AAT ATG ACC TTC CTG AAG AAC ATT GAC          942
Tyr Ile Ile Gly Thr Ala Leu Asn Met Thr Phe Leu Lys Asn Ile Asp
    65                  70                  75

CTA  G GTGCTTATTC TGCAGACCCA ATCAAGGTGA CCATTGACTA ACCTTATGGT            996
Leu
 80

TCAACAG  AG GTC CTC GGA ACA ATC TTA TTC ACT AAC GAT ACA GAC TAC         1044
        Glu Val Leu Gly Thr Ile Leu Phe Thr Asn Asp Thr Asp Tyr
                    85                  90

TGG CAA GCC AAC TCC TTC AAA CAG GGC TTC CAG AAC GCT ACG ACC TTC         1092
Trp Gln Ala Asn Ser Phe Lys Gln Gly Phe Gln Asn Ala Thr Thr Phe
 95                 100                 105                 110

TTC CAA CTC GGT GGT GAA GAT GTG AAT ATG TAC GGT GGT GGT ACA ATC         1140
Phe Gln Leu Gly Gly Glu Asp Val Asn Met Tyr Gly Gly Gly Thr Ile
                115                 120                 125

AAT GGC AAC GGA CAG GTC TGG TAT GAT CTG TAT GCC GAA GAT GAT CTC         1188
Asn Gly Asn Gly Gln Val Trp Tyr Asp Leu Tyr Ala Glu Asp Asp Leu
            130                 135                 140

ATT CTG CGT CCC ATC TTG ATG GGC ATC ATT GGG CTG AAT GGT GGC ACA         1236
Ile Leu Arg Pro Ile Leu Met Gly Ile Ile Gly Leu Asn Gly Gly Thr
        145                 150                 155

ATT GGT CCG CTG AAG CTG CGG TAC TCG CCG CAA TAC TAC CAT TTT GTG         1284
Ile Gly Pro Leu Lys Leu Arg Tyr Ser Pro Gln Tyr Tyr His Phe Val
    160                 165                 170

GCT AAC TCG TCG AAT GTG CTC TTT GAC GGG ATT GAC ATT TCG GGT TAT         1332
Ala Asn Ser Ser Asn Val Leu Phe Asp Gly Ile Asp Ile Ser Gly Tyr
175                 180                 185                 190

AGT AAG AGC GAC AAC GAA GCC AAA AAC ACT GAT GGA  TG  GTGCGTTTTA         1380
Ser Lys Ser Asp Asn Glu Ala Lys Asn Thr Asp Gly  Trp
                195                 200

TCCTGCTTTA CACTGAGCGT TATACTGACC TTTTTCCCGT AG G GAT ACC TAC CGC        1435
                                                Asp Thr Tyr Arg
                                                    205
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TCG AAC AAT ATC GTT ATC CAG AAT TCG GTG ATC AAC AAC GGT GAT  G          1481
Ser Asn Asn Ile Val Ile Gln Asn Ser Val Ile Asn Asn Gly Asp
        210                 215                 220

GTAAGTTAAA CCTAAGTAGC GTCATACTTC AACAATTCTA ACCTGCAAAC CTACACAG  AC  1541
                                                                 Asp


TGT GTC TCT TTC AAG CCG AAC AGC ACC AAT ATC CTC GTT CAG AAC CTT      1589
Cys Val Ser Phe Lys Pro Asn Ser Thr Asn Ile Leu Val Gln Asn Leu
    225                 230                 235

CAC TGC AAT GGC TCC CAC GGC ATT TCT GTT GGC TCT CTC GGC CAA TAC      1637
His Cys Asn Gly Ser His Gly Ile Ser Val Gly Ser Leu Gly Gln Tyr
240                 245                 250                 255

AAG GAT GAG GTT GAC ATC GTT GAG AAT GTC TAT GTG TAC AAC ATC TCT      1685
Lys Asp Glu Val Asp Ile Val Glu Asn Val Tyr Val Tyr Asn Ile Ser
                260                 265                 270

ATG TTT AAT GCT TCG GTA TGT CTG AAC TTT AAC CAT ATA ATA GAC TTC      1733
Met Phe Asn Ala Ser Val Cys Leu Asn Phe Asn His Ile Ile Asp Phe
            275                 280                 285

TTA CTA ACT TGG TTG CAG GAT ATG GCC CGC ATC AAG GTT TGG CCT GGT      1781
Leu Leu Thr Trp Leu Gln Asp Met Ala Arg Ile Lys Val Trp Pro Gly
        290                 295                 300

ACT CCC TCT GCG CTA TCT GCC GAT CTT CAA GGC GGC GGT GGC TCG GGT      1829
Thr Pro Ser Ala Leu Ser Ala Asp Leu Gln Gly Gly Gly Gly Ser Gly
    305                 310                 315

AGC GTA AAG AAT ATC ACC TAT GAC ACC GCA CTC ATT GAT AAT GTC GAC      1877
Ser Val Lys Asn Ile Thr Tyr Asp Thr Ala Leu Ile Asp Asn Val Asp
320                 325                 330                 335

TGG GCC ATT GAA ATC ACG CAG TGC TAT GGG CAG AAG AAT ACT ACC TTG      1925
Trp Ala Ile Glu Ile Thr Gln Cys Tyr Gly Gln Lys Asn Thr Thr Leu
                340                 345                 350

TGC AAC GAG TAC CCG GTTAGTAGAC CTTCAGCCGC TTTCCCGAAG CTATCCTAAT      1980
Cys Asn Glu Tyr Pro
            355

ACAATAATAG AGC TCT CTC ACC ATC TCG GAC GTC CAC ATC AAG AAC TTC        2029
          Ser Ser Leu Thr Ile Ser Asp Val His Ile Lys Asn Phe
                    360                 365

CGC GGT ACG ACG TCG GGA TCG GAA GAT CCC TAT GTT GGG ACA ATT GTT      2077
Arg Gly Thr Thr Ser Gly Ser Glu Asp Pro Tyr Val Gly Thr Ile Val
370                 375                 380                 385

TGT TCC AGT CCT GAT GTAAGTGCCC TCCAGGATAT GCGTTTAGTG TGCAATGGCT      2132
Cys Ser Ser Pro Asp
                390
```

GACACTCGAT AG ACT TGC TCG GAT ATC TAT ACT TCC AAT ATT AAT GTA 2180
Thr Cys Ser Asp Ile Tyr Thr Ser Asn Ile Asn Val
395 400

ACA AGC CCG GAT GGA ACC AAC GAC TTT GTT TGC GAT AAT GTGAGTCGNC 2229
Thr Ser Pro Asp Gly Thr Asn Asp Phe Val Cys Asp Asn
405 410 415

CAAGGCCAGG TGAAGATCTA AATCGGTGAC TAACGCTGTC CAG GTC GAT GAG AGT 2284
Val Asp Glu Ser

CTT CTG AGT GTC AAC TGC ACC GCC ACT TCT GAT TAAGTTAGAA TGCCTGTTAA 2337
Leu Leu Ser Val Asn Cys Thr Ala Thr Ser Asp
420 425 430

CATGTACACT GGAGGTCAGG CCTTACTTAG ACGGATGAAG GGTGTATATA TTCACTTTGG 2397

GTTCGGCTGT GTATATACGT GAGCAAATAA TATCTGTATG CTTTTGACTT AGGGAAGGTC 2457

AGGCGATCAA GTAACTTTCT GGTTCAAACC TTGATCATGG TTCTCAGTAG ACGCTCCTTC 2517

TGCTTTTTGC GTTGCGATAC GTCACATGCC GAGGGAAGAA TCATTGCTTC CAGGTGCTTG 2577

CGCTCTCCTG AGTGATACTG CACTTGCTGT CCAGGATACT ACATCTCTCT GCATTTACCT 2637

GCTGTTCATC AAACAAGCAA GCAACCTGAA GAGCCACCTA CCAAGTATAG TAGGATCTAG 2697

AGCGCTCCGA GGAGATCGTG TAGACCATAC TAGATCCTCG AAAATGTAAA TTGCAAACCA 2757

TATTTCAATT CTAATTCATC TGAAGTCTGG CAGCGAGATA TATAACGGAA GTCTCTTGGA 2817

ACACGAACCC TCGGCTGATG ATCGTGACCT CCTGAGTAGC ACCCAACCAG CGGACTACAA 2877

GATGGACAAC GAACCTTACC CACAACCCTT AAGTACTTTG GAACCAAGTG GCAATGATTT 2937

GTATTCTTTA TCGGTGTGCG TCATGCACCT CCTGCAG 2974

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 452 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Arg Leu Thr His Val Leu Ser His Thr Leu Gly Leu Leu Ala Leu
-22 -20 -15 -10

Gly Ala Thr Ala Glu Ala Phe Ser Arg Ser Arg Glu Ala Ala Cys Gly
-5 1 5 10

Pro Lys Lys Pro Phe Arg Pro Leu Pro Thr Ser Gln Ser Arg Asp Lys

```
                                15                  20                  25
Thr Cys His Val Arg Ser His Gly Asp Gly Thr Asp Asp Ser Asp Tyr
            30                  35                  40
Ile Leu Ser Ala Leu Asn Gln Cys Asn His Gly Gly Lys Val Val Phe
        45                  50                  55
Asp Glu Asp Lys Glu Tyr Ile Ile Gly Thr Ala Leu Asn Met Thr Phe
    60                  65                  70
Leu Lys Asn Ile Asp Leu Glu Val Leu Gly Thr Ile Leu Phe Thr Asn
75                  80                  85                  90
Asp Thr Asp Tyr Trp Gln Ala Asn Ser Phe Lys Gln Gly Phe Gln Asn
                95                  100                 105
Ala Thr Thr Phe Phe Gln Leu Gly Gly Glu Asp Val Asn Met Tyr Gly
            110                 115                 120
Gly Gly Thr Ile Asn Gly Asn Gly Gln Val Trp Tyr Asp Leu Tyr Ala
        125                 130                 135
Glu Asp Asp Leu Ile Leu Arg Pro Ile Leu Met Gly Ile Ile Gly Leu
    140                 145                 150
Asn Gly Gly Thr Ile Gly Pro Leu Lys Leu Arg Tyr Ser Pro Gln Tyr
155                 160                 165                 170
Tyr His Phe Val Ala Asn Ser Ser Asn Val Leu Phe Asp Gly Ile Asp
                175                 180                 185
Ile Ser Gly Tyr Ser Lys Ser Asp Asn Glu Ala Lys Asn Thr Asp Gly
            190                 195                 200
Trp Asp Thr Tyr Arg Ser Asn Asn Ile Val Ile Gln Asn Ser Val Ile
        205                 210                 215
Asn Asn Gly Asp Asp Cys Val Ser Phe Lys Pro Asn Ser Thr Asn Ile
    220                 225                 230
Leu Val Gln Asn Leu His Cys Asn Gly Ser His Gly Ile Ser Val Gly
235                 240                 245                 250
Ser Leu Gly Gln Tyr Lys Asp Glu Val Asp Ile Val Glu Asn Val Tyr
                255                 260                 265
Val Tyr Asn Ile Ser Met Phe Asn Ala Ser Val Cys Leu Asn Phe Asn
            270                 275                 280
His Ile Ile Asp Phe Leu Leu Thr Trp Leu Gln Asp Met Ala Arg Ile
        285                 290                 295
Lys Val Trp Pro Gly Thr Pro Ser Ala Leu Ser Ala Asp Leu Gln Gly
    300                 305                 310
Gly Gly Gly Ser Gly Ser Val Lys Asn Ile Thr Tyr Asp Thr Ala Leu
315                 320                 325                 330
```

Ile Asp Asn Val Asp Trp Ala Ile Glu Ile Thr Gln Cys Tyr Gly Gln
335 340 345

Lys Asn Thr Thr Leu Cys Asn Glu Tyr Pro Ser Ser Leu Thr Ile Ser
350 355 360

Asp Val His Ile Lys Asn Phe Arg Gly Thr Thr Ser Gly Ser Glu Asp
365 370 375

Pro Tyr Val Gly Thr Ile Val Cys Ser Ser Pro Asp Thr Cys Ser Asp
380 385 390

Ile Tyr Thr Ser Asn Ile Asn Val Thr Ser Pro Asp Gly Thr Asn Asp
395 400 405 410

Phe Val Cys Asp Asn Val Asp Glu Ser Leu Leu Ser Val Asn Cys Thr
415 420 425

Ala Thr Ser Asp
430

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: pki SmaI sense

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TTTTCCTTCC CGGGCAC 17

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: pki-pgaX antisense

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTGCGTGAGT CTCATCTTGA CGGATGATTG 30

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: pki-pgaX sense

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CAATCATCCG TCAAGATGAG ACTCACGCAC 30

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: pgaX NcoI antisense

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AGTGCCATCT CCATGGC 17

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: 3062

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: one-of(3, 6, 9, 15)
(D) OTHER INFORMATION: /note= "N at position 3, 6, 9, and 15 is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGNGTNCCNG GVCANACYTT DAT 23

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: 3063

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: one-of(9, 15, 18)
(D) OTHER INFORMATION: /note= "N at position 9, 15, and 18 is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATHAARGTNT GBCCNGGNAC NCC 23

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: HR7734

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: one-of(3, 9, 12, 15, 21, 23)
(D) OTHER INFORMATION: /note= "N at position 3, 9, 12, 15, 21, and 23 is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGNCCDATNG TNCCNCCRTT NARNCCDATD AT 32

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: 3059

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: one-of(9, 12, 18, 21, 24)
(D) OTHER INFORMATION: /note= "N at position 9, 12, 18, 21, and 24 is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ATHATHGGNY TNAAYGGNGG NACNATHGG 29

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: 3060

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: one-of(6, 9, 12, 15)
(D) OTHER INFORMATION: /note= "N at position 6, 9, 12, and 15 is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GAGGCNGCNA CNGGNCCNAA GAA 23

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: 3061

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: one-of(6, 9, 15)
(D) OTHER INFORMATION: /note= "N at position 6, 9, and 15

is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TTYTTNGGNC CRTTNGCNGC YTC 23

Claims

1. A purified and isolated DNA sequence which encodes a polypeptide having the activity of the Aspergillus tubigensis PGX enzyme, characterized in that the DNA sequence comprises,

a) the sequence of SEQ ID No.1,

b) genetic variants of the sequence of a),

c) a DNA sequence capable of hybridizing with the sequences of a) or b) or with parts thereof.

2. A DNA construct containing a DNA sequence according to claim 1, operably linked to regulatory regions capable of directing the expression of said DNA sequence in a suitable host organism.

3. A DNA vector containing the construct of claim 2.

4. A transformed microbial host cell capable of expressing a gene encoding a protein having exo-polygalacturonase activity characterized in that the host cell contains a DNA vector according to claim 3.

5. A method for obtaining expression of exo-polygalacturonase comprising, culturing the microbial host cell of claim 4 under conditions wherein the cloned gene is expressed.

6. A method for obtaining exo-polygalacturonase comprising the steps of

a) culturing the microbial host cell of claim 4 under conditions giving rise to the expression of the DNA sequence of claim 1,

b) recovering the polypeptide having PGX activity.

7. A polypeptide having PGX activity characterized in that the polypeptide is produced by the method of claim 6.

8. Use of a polypeptide obtained according to claim 7 and having exo-polygalacturonase activity to degrade a polymer containing glucuronic acid containing residues.

9. Purified and isolated expression and transcription regulatory regions as found in the 5'non-coding sequences of the Aspergillus tubigensis pgaX gene.

10. pIM362

FIGURE 1

a:

```
                    A     C  T
5' GGIGTICCIGGCCAIACTTTAAT 3'
                    G        G
```

(3062) (non-coding strand)

```
   C  G     T         A
5' ATTAAAGTITGGCCIGGIACGCC 3'
   A        C         C
                      T
```

(3063) (coding strand)

b:

```
        T                          T  T
5' GGICCAATIGTICCICCATTIAAICCAATAAT 3'
        G            G    G   G  G
```

(7734) (non-coding strand)

```
   A  A                       A
5' ATTATTGGITTIAATGGIGGIACIATTGGICC 3'
   C  C   C    C              C
```

(3059) (coding strand)

c:

```
                      A  A
5' GAAGCIGCIAAIGGICCCAAGAA 3'
   G       C      G
                  T
```

(3060) (coding strand)

```
    T        A        C
5' TTCTTIGGICCGTTIGCIGCTTC 3'
```

(3061) (non-coding strand)

a: derived from CB 1, residues 3-10
b: derived from CB 2, residues 2-12
c: derived from CB 3, residues 6-13

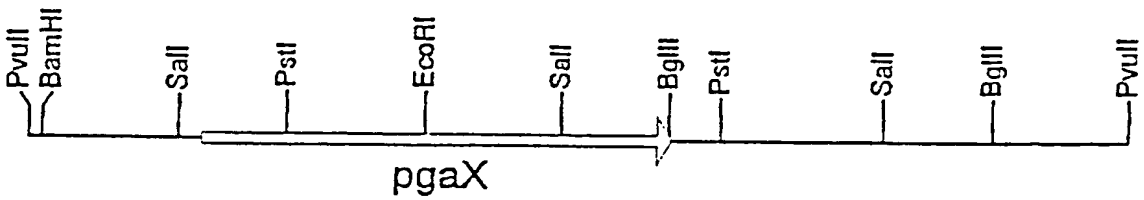


Figure 2

FIGURE 3

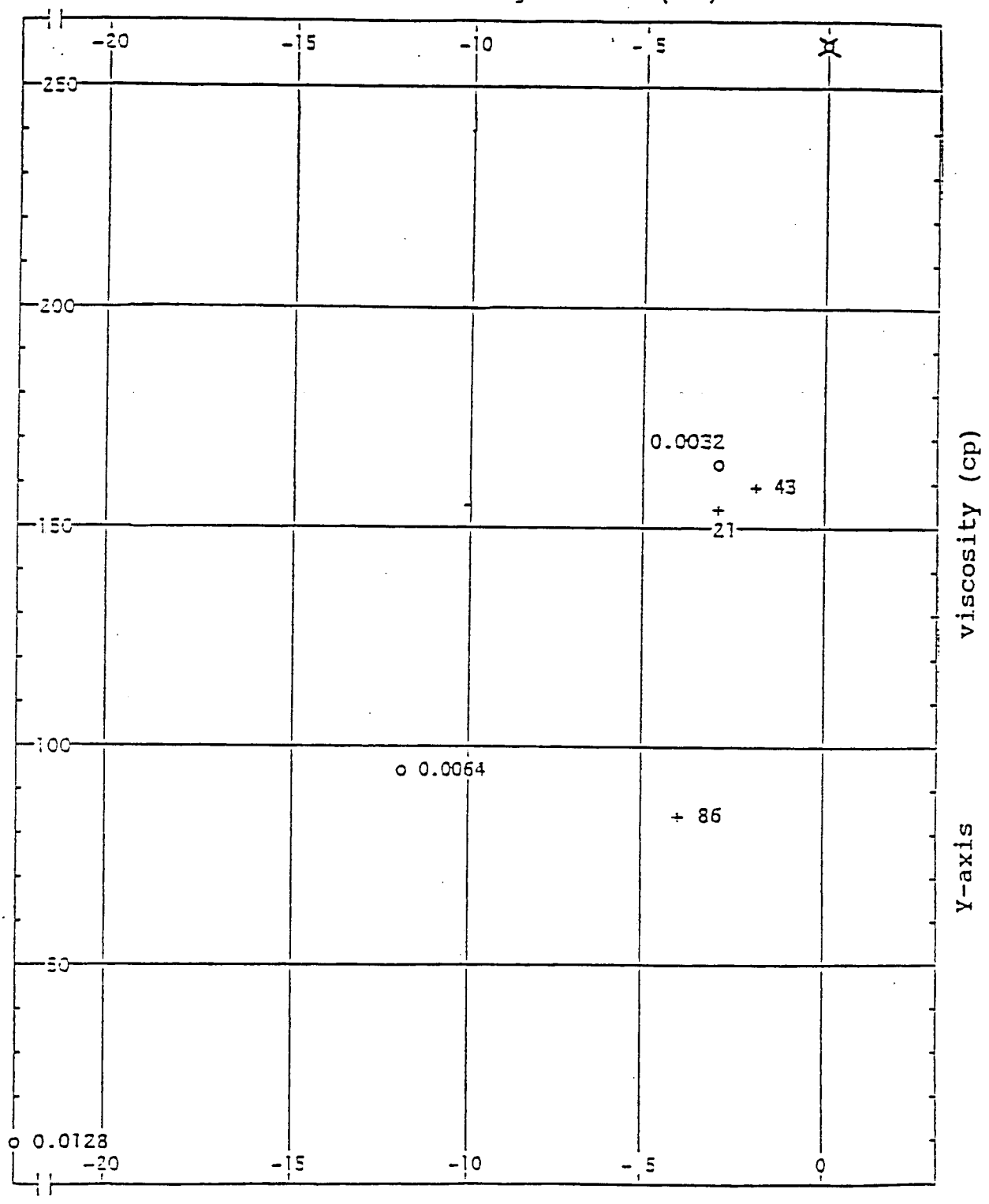
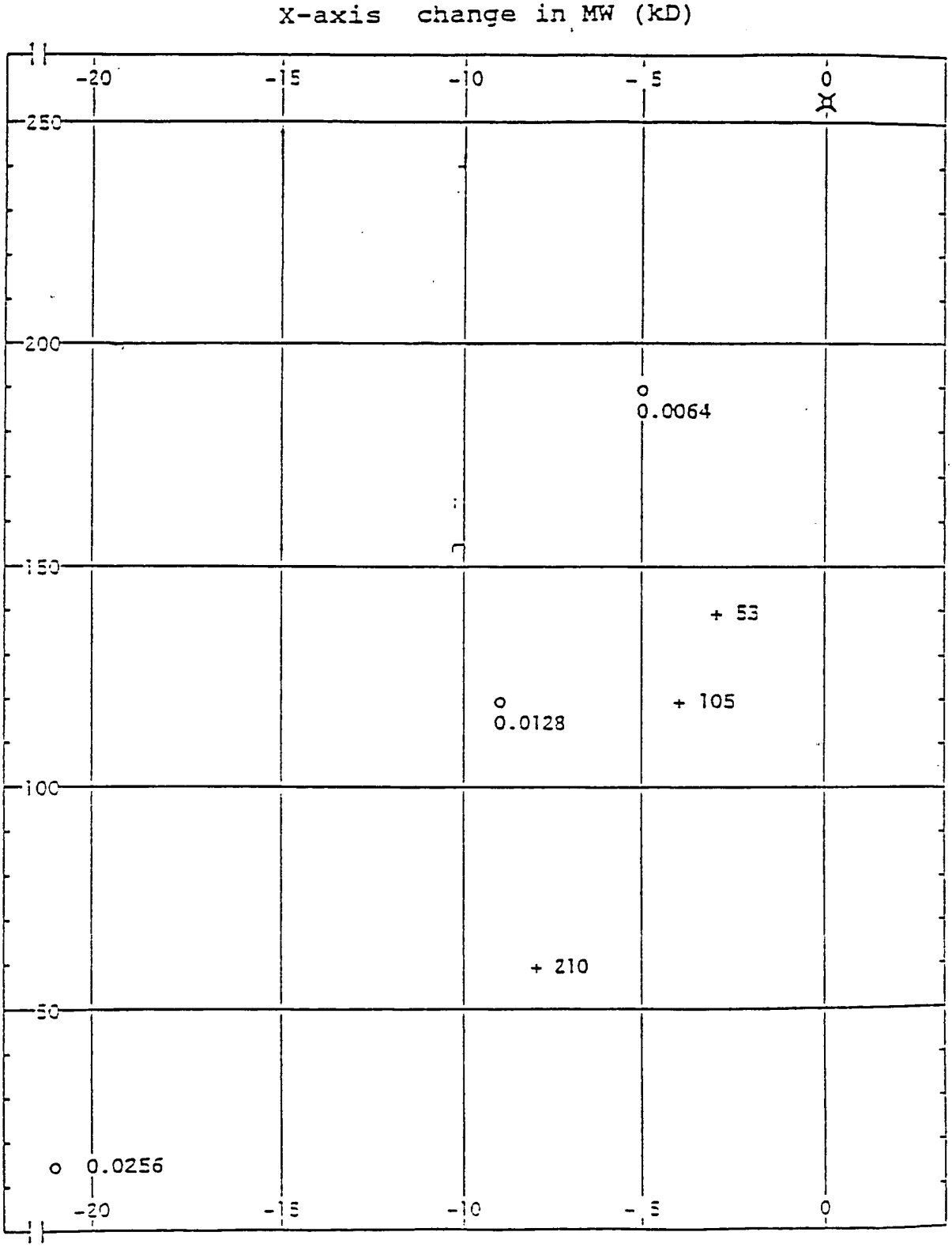


FIGURE 4



INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/EP 93/03704

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/56 C12N15/11 C12N9/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 421 919 (CYBA-GEIGY AG) 10 April 1991 cited in the application see page 4, line 2 - line 14 see page 5, line 12 - line 33 see page 5, line 43 - page 6, line 15 see page 8, line 17 - line 21 ---	1-10
A	WO,A,91 04331 (GENESIT OY) 4 April 1991 see page 4, line 30 - page 5, line 12 see page 10, line 7 - page 11, line 29 --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 March 1994	25. 94 04

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Montero Lopez, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CURRENT GENETICS vol. 19, no. 6 , 1991 pages 467 - 474 HENDRIK J.D. BUSSINK ET AL. 'Expression and sequence comparison of the Aspergillus niger and Aspergillus tubigensis genes encoding polygalacturonase II' see page 467, right column, last paragraph see page 468, right column, paragraph 1 see page 469, left column, paragraph 3; figure 5 -----	1-5

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte: nal Application No
PCT/EP 93/03704

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0421919	10-04-91	AU-B-	640405	26-08-93
		AU-A-	6205490	07-03-91
		JP-A-	3224489	03-10-91
WO-A-9104331	04-04-91	AU-B-	644101	02-12-93
		AU-A-	6330590	18-04-91
		EP-A-	0491743	01-07-92
		JP-T-	5500309	28-01-93